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I HEREBY CERTIFY that annexed hereto is a true copy of documents filed in connection with the following patent application:

Application No.	1999/0782
Date of Filing	20 September 1999
Applicant	ENTERPRISE IRELAND (trading as Bioresearch Ireland) an Irish agency established by statue of Glasnevin, Dublin 9, Ireland.and NATIONAL UNIVERSITY OF IRELAND CORK, an Irish body established by the Universities Act 1997 of College Road, Cork, Ireland

Dated this 9 day of December 2003.

An officer authorised by the
Controller of Patents, Designs and Trademarks.

FORM NO. 1

990782

REQUEST FOR THE GRANT OF A PATENT

PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)

__X__ the grant of a patent under Part II of the Act

_____ the grant of a short-term patent under Part III of the Act on the basis of the information furnished hereunder.

1. Applicant(s)

Name Enterprise Ireland (trading as Bioresearch Ireland)
Address Glasnevin, Dublin 9, Ireland.

Description/Nationality
An Irish agency established by statue

Name National University of Ireland, Cork.
Address College Road, Cork, Ireland

Description/Nationality
A Irish body established by the Universities Act 1997

2. Title of Invention

"Use of *Lactobacillus Salivarius*"

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

Previous filing date	Country in or for which filed	Filing No.

4. Identification of Inventor(s)

Name(s) of person(s) believed by Applicants(s) to be the inventor(s)

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Address: Seafort, Fort Hill, Kinsale, County Cork, Ireland.

990782

5. Statement of right to be granted a patent (Section 17(2) (b)

The Applicant derives the rights to the Invention by virtue of a Deed of Assignment dated September 20, 1999.

6. Items accompanying this Request – tick as appropriate

(i) ___X___ Prescribed filing fee (£100.00)

(ii) ___X___ Specification containing a description and claims

_____ Specification containing a description only

___X___ Drawings referred to in description or claims

(iii) _____ An abstract

(iv) _____ Copy of previous application (s) whose priority is claimed

(v) _____ Translation of previous application whose priority is claimed

(vi) _____ Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant (s))

7. Divisional Application (s)

The following information is applicable to the present application which is made under Section 24 –

Earlier Application No: .....

Filing Date: .....

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name	Address
John A. O'Brien & Associates	The address recorded for the time being in the Register of Patent Agents, and currently Third Floor, Duncairn House, 14 Carysfort Avenue, Blackrock, Co. Dublin, Ireland.

9. Address for Service (if different from that at 8)

As above

Signed _____ JOHN A. O'BRIEN & ASSOCIATES

Date September 20, 1999

"Use of *Lactobacillus salivarius*

Introduction

Both immunological and non-immunological defence mechanisms protect the human gastrointestinal tract from colonisation by intestinal bacteria (1). Innate defence mechanisms include the low pH of the stomach, bile salts, peristalsis, mucin layers and anti-microbial compounds such as lysozyme (2). Immunological mechanisms include specialised lymphoid aggregates, underlying M cells, called peyers patches which are distributed throughout the small intestine and colon (3). Luminal antigens presented at these sites result in stimulation of appropriate T and B cell subsets with establishment of cytokine networks and secretion of antibodies into the gastrointestinal tract (4, 5). In addition, antigen presentation may occur via epithelial cells to intraepithelial lymphocytes and to the underlying lamina propria immune cells (6). Therefore, the host invests substantially in immunological defense of the gastrointestinal tract. However, as the gastrointestinal mucosa is the largest surface at which the host interacts with the external environment, specific control mechanisms must be in place to regulate immune responsiveness to the 100 tons of food which is handled by the gastrointestinal tract over an average lifetime (7). Furthermore, the gut is colonised by over 500 species of bacteria numbering 10^{11}-10^{12}/g in the colon. Thus, these control mechanisms must be capable of distinguishing non-pathogenic adherent bacteria from invasive pathogens which would cause significant damage to the host. In fact, the intestinal flora contributes to defense of the host by competing with newly ingested potentially pathogenic micro-organisms. Furthermore, consumption of non-pathogenic, or probiotic, bacteria has resulted in enhancement of immune parameters in healthy volunteers. Examples of these immune modulatory effects can be observed in Table 1.

The enteric flora are important to the development and proper function of the intestinal immune system. In the absence of an enteric flora, the intestinal

immune system is underdeveloped, as demonstrated in germ free animal models, and certain functional parameters are diminished, such as macrophage phagocytic ability and immunoglobulin production (8, 9). The importance of the gut flora in stimulating non-damaging immune responses is becoming more evident. The increase in incidence and severity of allergies in the western world has been linked with an increase in hygiene and sanitation, concomitant with a decrease in the number and range of infectious challenges encountered by the host. This lack of immune stimulation may allow the host to react to non-pathogenic, but antigenic, agents resulting in allergy or autoimmunity.

Table 1. Immune Enhancing Effects Following Oral Consumption of Probiotic Bacteria.

Observed Effect	Reference
Increased Macrophage Phagocytosis	10
Increased Natural Killer Cell Activity	11
Increased IFNγ serum levels	12
Increased B cell and NK cell numbers	12
Promotion of IgA responses	11, 13-15
Increased DTH responses	16

The deliberate consumption of non-pathogenic bacteria, such as probiotic bacteria, may provide a health promoting immune challenge to the host. The promotion of immunoglobulin secretion into the lumen, following consumption of probiotic bacteria (13), may bind allergens and prevent their recognition by the host. In addition, interaction with certain intraepithelial lymphocyte subsets may suppress immune responses to allergens within the gastrointestinal tract possibly resulting in tolerance to oral and inhaled antigens (17). Inadequate priming of T cell subsets may result in an incorrect cytokine balance or may contribute to a

failure of the T cell repertoire to recognise epitopes that are crossreactive between self and nonself (18).

The majority of pathogenic organisms gain entry via mucosal surfaces. Efficient vaccination of these sites protects against invasion by a particular infectious agent. Oral vaccination strategies have concentrated, to date, on the use of attenuated live pathogenic organisms or purified encapsulated antigens (19, 20). Murine studies have demonstrated that consumption of probiotic bacteria expressing foreign antigens can elicit protective immune responses. The gene encoding tetanus toxin fragment C (TTFC) was expressed in *Lactococcus lactis* and mice were immunised via the oral route. This system was able to induce antibody titres significantly high enough to protect the mice from lethal toxin challenge (21). In addition to antigen presentation, live bacterial vectors can produce bioactive compounds, such as immunostimulatory cytokines, *in vivo*. *L. lactis* secreting bioactive human IL-2 or IL-6 and TTFC induced 10-15 fold higher serum IgG titres in mice immunised intranasally (22). However, with this particular bacterial strain, the total IgA level was not increased by coexpression with these cytokines. Other bacterial strains, such as *Streptococcus gordonii*, are also being examined for their usefulness as mucosal vaccines. Recombinant *S. gordonii* colonising the murine oral and vaginal cavities induced both mucosal and systemic antibody responses to antigens expressed by this bacterial (23, 24). Thus oral immunisation using probiotic bacteria as vectors have been shown to evoke an immune response.

Aberrant immune responses to the indigenous microflora have been implicated in certain disease states, such as inflammatory bowel disease (25). Antigens associated with the normal flora usually lead to immunological tolerance and failure to achieve this tolerance is a major mechanism of mucosal inflammation (26). Evidence for this breakdown in tolerance includes an increase in antibody levels directed against the gut flora in patients with IBD. In addition, certain mouse models predisposed to inflammatory lesions in the gastrointestinal tract

remain disease free when housed in germ free conditions or when treated with antibiotics (27, 28). In a recent clinical trial where pouchitis patients consumed a cocktail of probiotic bacteria, only 15% of the test group relapsed compared to a 100% relapse rate in the placebo group (29).

Statements of Invention

The invention relates to strains of *Lactobacillus salivarius*, especially those isolated from resected and washed human gastrointestinal tract which inhibits a broad range of Gram positive and Gram negative micro-organisms and which secretes a product having antimicrobial activity into a cell – free supernantant, said activity being produced only by growing cells and being destroyed by proteinase K and pronase E, the inhibitory properties of said strain and its seretory products being maintained in the presence of physiological concentration of human bile and human gastric juice.

Such strains of *Lactobacillus salivarius* are disclosed in WO 98/35014.

An especially preferred strain of *Lactobacillus salivarius* is *Lactobacillus salivarius* strain UCC 118 or a mutant or variant thereof.

A deposit of *Lactobacillus salivarius* strain UCC 118 was made at the NCIMB on November 27, 1996 and accorded the accession number NCIMB 40829.

The invention provides the use of a strain of *Lactobacillus salivarius* as an anti-inflammatory agent.

In one aspect the invention provides the use of a strain of *Lactobacillus salivarius* for prophylaxis and/or treatment of inflammatory bowel disease.

In another aspect the invention provides the use of a strain of *Lactobacillus salivarius* for the prophylaxis and/or treatment of gastro intestinal cancer(s).

In a preferred embodiment of the invention the strain of *Lactobacillus salivarius* is isolated from resected and washed human gastrointestinal tract which inhibits a groad range of Gram positive and Gram negative micro-organisms and which secretes a product having anti-microbial activity into a cell-free supernatant, said activity being produced only by growing cells and being destroyed by proteinase K and pronase E as described in WO98/35014.

In a particualry preferred embodiment the *Lactobacillus salivarius* strain is strain UCC 118 or a mutant or variant thereof.

Detailed Description

We have developed criteria for *in vitro* selection of probiotic bacteria that reflect certain *in vivo* effects on their host, such as modulation of the GIT microflora and modulation of the mucosal immune response resulting in the production of secretory antibodies specific to the consumed strain. *Lactobacillus salivarius* subsp. *salivarius* UCC118 survives passage through the gastrointestinal tract, adheres to human intestinal cell lines and is non-inflammatory.

Detailed description of the *In Vivo* demonstration of the anti-inflammatory effects of *Lactobacillus salivarius* especially subspecies *salivarius* UCC118.

Murine model of gastrointestinal inflammation

Aberrant immune responses to the indigenous microflora have been implicated in certain disease states, such as inflammatory bowel disease (25). Antigens associated with the normal flora usually lead to immunological tolerance and failure to achieve this tolerance is a major mechanism of mucosal inflammation (26). Evidence for this breakdown in tolerance includes an increase in antibody levels directed against the gut flora in patients with IBD. In addition, certain mouse models predisposed to inflammatory lesions in the gastrointestinal tract remain disease free when housed in germ free conditions or when treated with antibiotics (27, 28).

C57BL/6 Interleukin-10 knockout mice are predisposed to developing enterocolitis in the presence of an enteric bacterial flora. When maintained in germ free conditions, IL-10 knock out mice do not develop the disease (30). Since the pathogenesis of this disease has been linked with the enteric flora, elimination of specific components of this flora may have a beneficial effect on the severity of this disease.

Lactobacillus salivarius subsp. *salivarius* UCC118 is a probiotic bacteria, which was isolated from a healthy human ileum. It is suited to gastrointestinal colonization as it fulfills many criteria set down for the selection of probiotic strains. These include traits such as bile tolerance, acid resistance and *in vitro* adherence to human colonic cell lines. Feeding trials in healthy humans have been conducted and considerable modification of the gastrointestinal flora was noted. In addition, UCC118 was perceived by the mucosal immune system resulting in the production and secretion of IgA specific to UCC118.

Thus, UCC118 survives passage through the gastrointestinal tract, modulates the gut flora and is perceived by the mucosal immune system. The influence of this probiotic bacteria in modulating inflammatory responses within the gastrointestinal tract was examined using a murine model of enterocolitis. In addition, we examined the role of *Lactobacillus salivarius* subsp. *salivarius* UCC118 in reducing the rate of neoplastic change within the gastrointestinal tract.

Twenty IL-10KO mice were studied (ten consumed probiotic organisms in milk and 10 consumed unmodified milk) for 16 weeks. Fecal microbial analysis was performed weekly to enumerate excretion of lactobacilli, *Clostridium perfringens*, bacteroides, coliforms, bifidobacteria and enterococci. At sacrifice, small and large bowel were microbiologically and histologically assessed.

Fecal coliform and enterococci levels were significantly reduced in test animals compared to the controls. At sacrifice, a significant reduction in *C. perfringens* numbers was observed in the test mice (Figure 1). There were no fatalities in the test group compared to two deaths from fulminant colitis in the control group. Only one test mouse developed colonic adenocarcinoma compared to five in the control group. Test animal mucosal inflammation consistently scored lower than that of the control mice (Figure 2). The reduction in tumour incidence following consumption of UCC118 may be related to the reduced level of inflammation within the gastrointestinal tract or may be due to elimination of pro-carcinogenic members of the gastrointestinal flora (31-33).

In this placebo controlled trial, modification of enteric flora in IL-10KO mice by probiotic lactobacilli was associated with reduced prevalence of colon cancer and mucosal inflammatory activity. Thus, UCC118 acts as a biotherapeutic agent for the treatment of gastrointestinal inflammation possibly due to its effect on the composition of the gut flora and activity of the immune system.

Human Trial with UCC118 in patients with active Crohn's disease.

Inflammatory bowel disease (IBD) encompasses a number of inflammatory disorders of the gastyrointestinal tract, including both Crohn's disease and Ulcerative colitis.

Patients suffering from active Crohn's disease have been treated with UCC118. Briefly, UCC118 was consumed in a fermented milk product for 6 weeks by 22 patients. Microbiological and immunological determinations were made at week 0, week 1, week 3 and week 6. This was not a placebo-controlled trial.

A number of systemic cytokine levels were measured over the course of feeding. In particular, tumour necrosis factor α (TNFα), a proinflammatory cytokine that has been implicated in the pathogenesis of many inflammatory disease states, including inflammatory bowel disease. Current therapies for inflammatory bowel disease specifically aim to reduce TNFα levels (34). In this trial, systemic TNFα levels were reduced following consumption of UCC118 (Figure 3).

In addition, patients were assessed regarding their Crohn's Disease Activity Index (CDAI) over the six week trial period. This index assesses the general health and well being of each patient (Figure 4). Overall, the disease activity index improved slightly for the majority of individuals in the trial. These are patients with moderately active disease and their CDAI scores would be expected to increase. However, following treatment with UCC118, CDAI scores did not increase and in fact they improved from a mean of 180 to 160.

Detailed description of the *In Vitro* demonstration of the mechanisms underlying the anti-inflammatory effects of *Lactobacillus salivarius* especially subspecies *salivarius* UCC118.

A number of methodologies have been utilised for these studies including ELISAs (extracellular protein determination), flow cytometry (intracellular protein determination) and cDNA expression arrays (mRNA expression). In particular, examination of the expression of tumour necrosis factor α has been targeted, due to its clinical importance, and suppression of the production of this cytokine, following exposure to UCC118, has been noted using all three methodologies.

Using a transwell assay system, with epithelial cells and peripheral blood mononuclear cells in the one compartment, extracellular cytokine levels were measured by ELISAs. Following co-incubation with UCC118, the amount of TNFα produced was significantly reduced compared to control cultures. Furthermore, IL-1RA and IFNγ levels dropped while IL-6 and soluble IL-6 receptor levels increased (Figure 5). Intracellular staining for TNFα confirmed the ELISA result as TNFα levels were lower in the UCC118 stimulated sample compared to controls.

Gene arrays measure the quantity of mRNA in a population of cells. We stimulated peripheral blood mononuclear cells with UCC118 for 24 hours and we examined the effect on cytokine gene expression (Figure 6). Considerable modification of cytokine gene expression was noted. For example, genes encoding the proinflammatory cytokines IL-1β and TNFα were turned off while genes encoding Th2 type cytokines, such as IL-6, were enhanced.

In vitro models have demonstrated that UCC118 is capable of inducing Th2 type cytokines (i.e. interleukin 6 and interleukin 6 soluble receptor) while suppressing the production of inflammatory cytokines such as tumour necrosis factor α and

interleukin 1 β. Thus, these results suggest that consumption of UCC118 would be of benefit to patients suffering from inflammatory diseases, such as IBD.

The invention is not limited to the embodiments hereinbefore described which may be varied in detail.

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Claims

1. Use of a strain of *Lactobacillus Salivarius* as an anti-inflammatory agent.

2. Use of a strain of *Lactobacillus Salivarius* for prophylaxis and/or treatment of inflammatory bowel disease.

3. Use of a strain of *Lactobacillus Salivarius* for the prophylaxis and/or treatment of gastro intestinal cancer(s).

4. Uses as claimed in any preceding claim wherein the strain of *Lactobacillus Salivarius* is isolated from resected and washed human gastrointestinal tract which inhibits a broad range of Gram positive and Gram negative micro-organisms and which secretes a product having anti-microbial activity into a cell-free supernatant, said activity being produced only by growing cells and being destroyed by proteinase K and pronase E.

5. Use as claimed in any preceding claim wherein the *Lactobacillus Salivarius* strain is strain UCC 118 or a mutant of variant thereof.

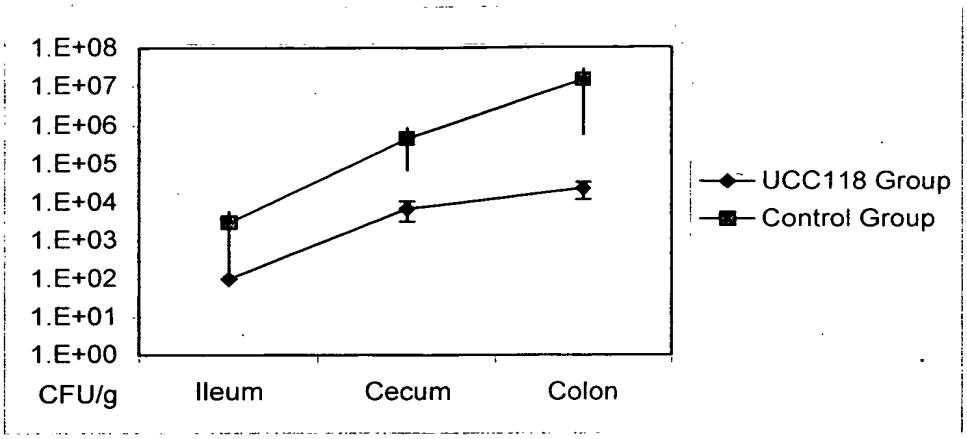

Figure 1. Following 16 weeks of feeding, all mice were sacrificed and their gastrointestinal contents were removed aseptically. *C. perfringens* was significantly reduced in the mice consuming UCC118 compared to the placebo group ($p<0.05$). Results are plotted as the mean log values $\pm$ standard error for each of the groups.

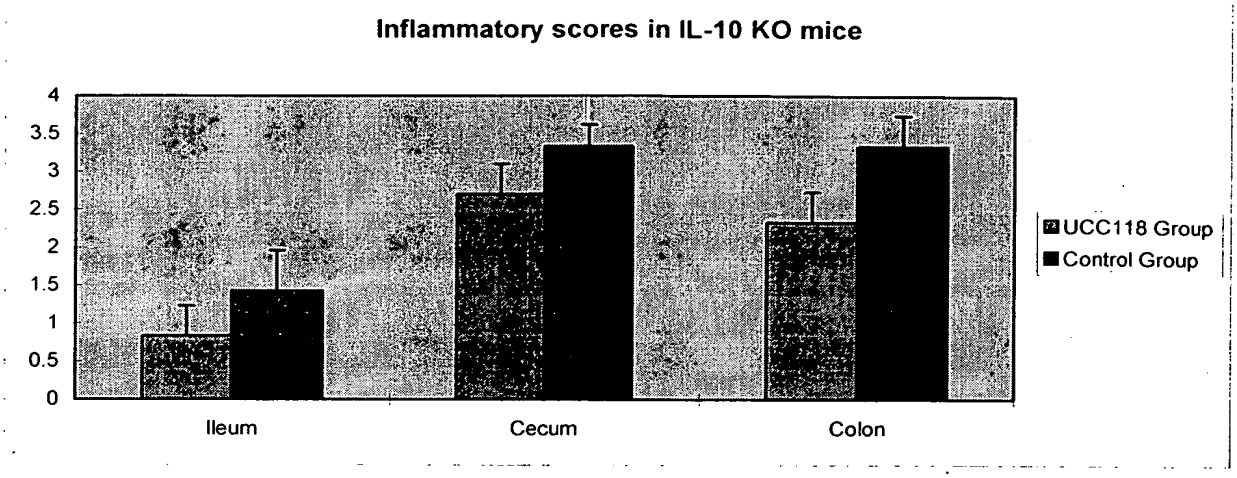

Figure 2. Inflammatory scores were graded by two independent histopathologists who were blinded to the identity of the mice. This is an arbitrary scoring system which takes a number of factors into account including inflammatory cell infiltrate, epithelial cell erosion and goblet cell depletion. Mice consuming UCC118 had consistently lower inflammatory scores at all three sites within the gastrointestinal tract. Results are shown as the mean $\pm$ standard error for each of the groups.

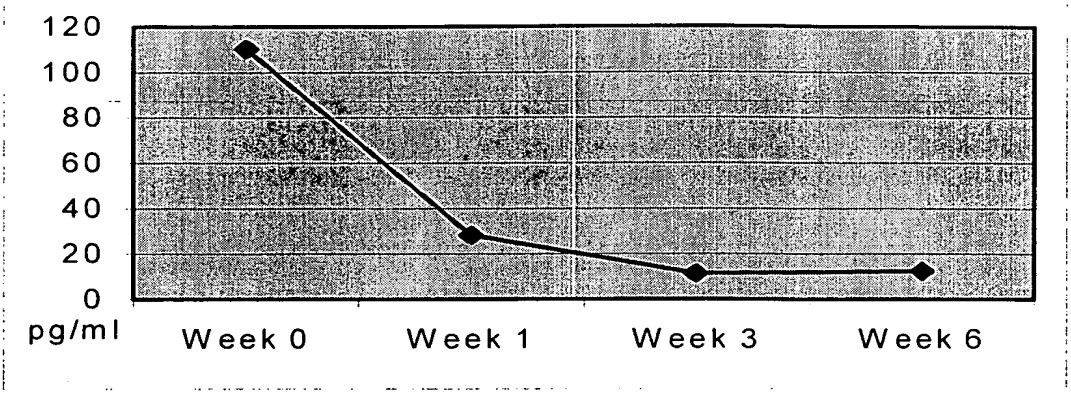

Figure 3. TNFα levels decrease over the six weeks that patients consume UCC118. Results are plotted as the mean pg/ml TNFα level for each time point (n=22).

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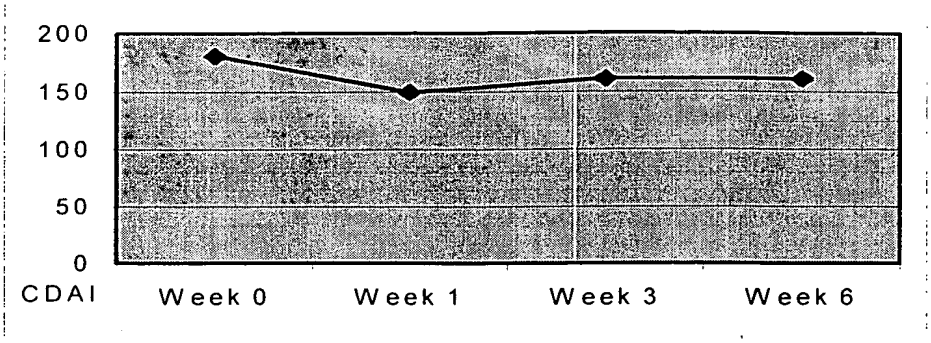

Figure 4. The mean CDAI scores for patients consuming UCC118 are shown over the course of probiotic feeding. CDAI scores decreased from an average of 180 to 160.

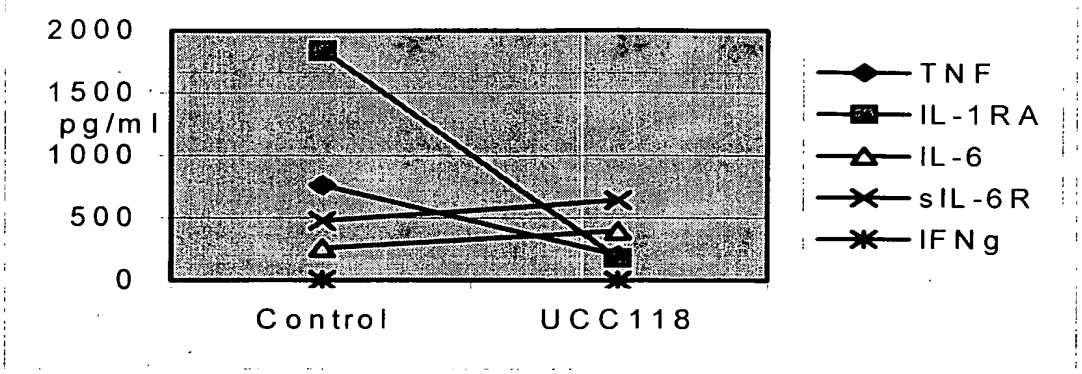

Figure 5. Using transwell assays, UCC118 was demonstrated to modulate cytokine production *in vitro*. Following exposure to UCC118, extracellular TNFα, IL-1RA and IFNγ levels decreased while Th2 type cytokines such as IL-6 and sIL-6R increased over control values. Results are expressed as pg/ml.

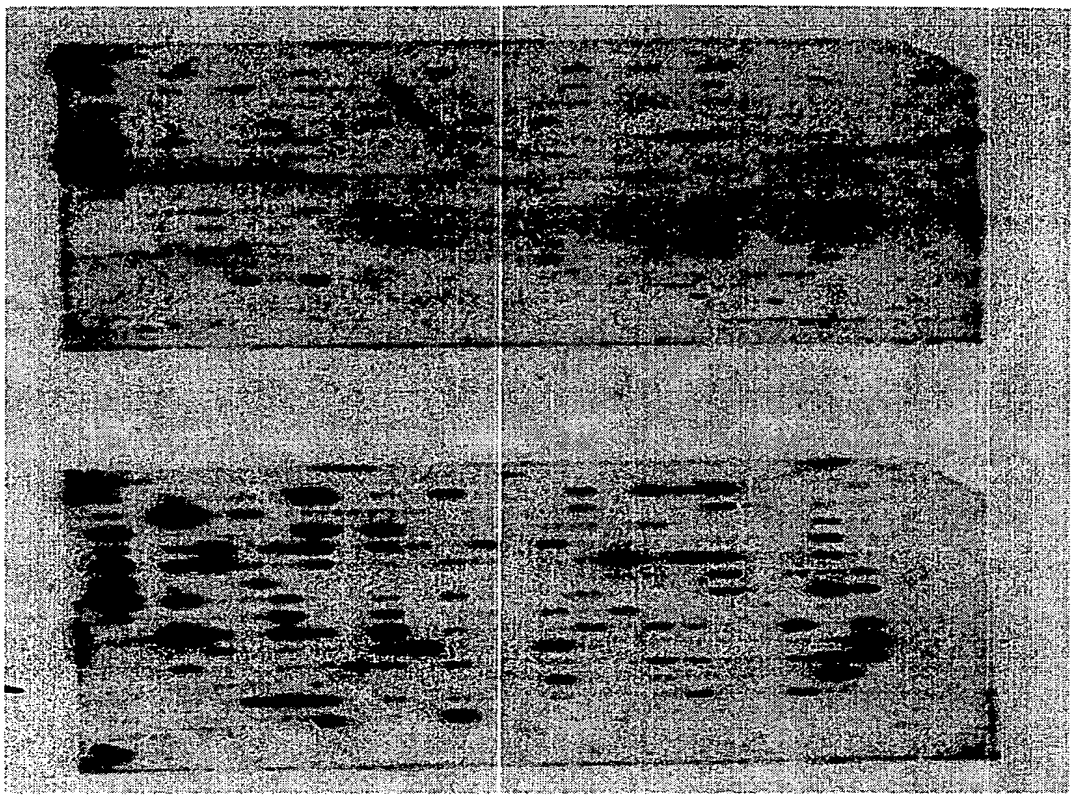

Figure 6. A gene array with specific gene sequences for 268 cytokines and related molecules was used to examine the immune response to UCC118. The bottom panel illustrates the control culture while the top panel illustrates cytokine gene expression by PBMCs following exposure to UCC118. Significant modulation of gene expression is evident.